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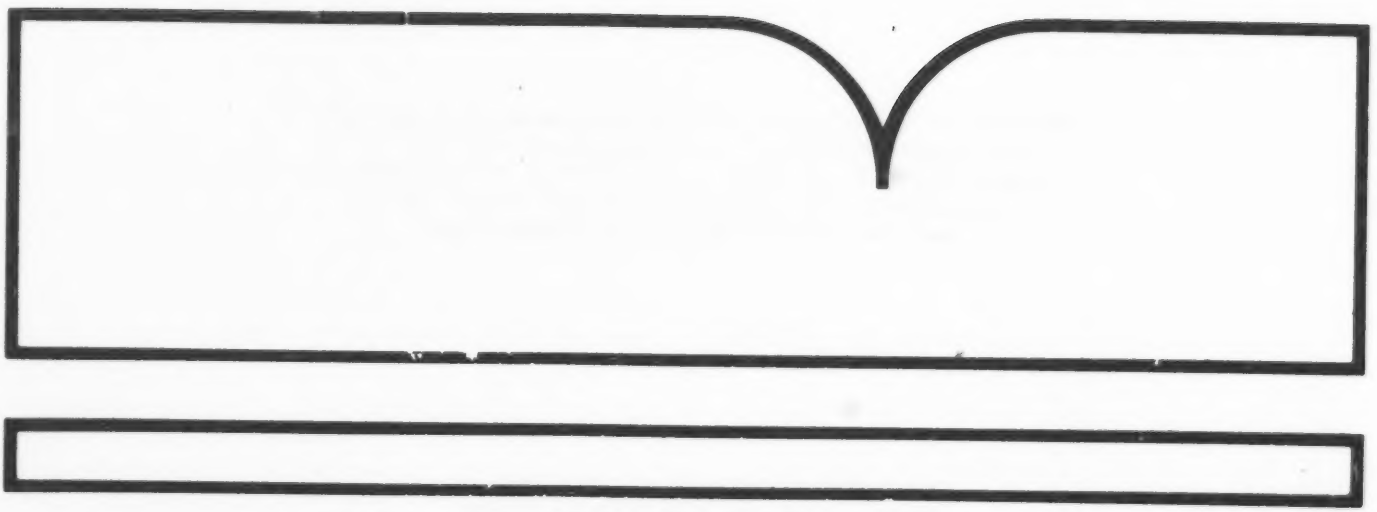

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SUBCELLULAR IONS BY X-RAY MICROANALYSIS FOR EVIDENCE OF HEPATOTOXICITY

by

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16. ABSTRACT

The hypothesis that the influx of extracellullar Ca^{2+} across a damaged plasma membrane is the final common pathway for chemically mediated cell death[1] has stirred considerable controversy and continued interest in the study of subcellular ion shifts as sensitive indicators of hepatotoxicity caused by environmental pollutants. A number of studies have shown that increases in hepatic mitochondrial calcium result from the ingestion of carbon tetrachloride. Quantitative measurement of calcium in subcellular components was accomplished in early studies by using techniques involving whole tissue homogenation and fractionation by centrifugation. In this study x-ray microanalysis of quick-frozen liver tissue, cryo-sectioned for the electron microscope, provided an alternative approach to studying subcellular ion distribution.

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The hypothesis that the influx of extracellular Ca^{2+} across a damaged plasma membrane is the final common pathway for chemically mediated cell death[1] has stirred considerable controversy and continued interest in the study of subcellular ion shifts as sensitive indicators of hepatotoxicity caused by environmental pollutants. A number of studies have shown that increases in hepatic mitochondrial calcium result from the ingestion of carbon tetrachloride. Quantitative measurement of calcium in subcellular components was accomplished in early studies by using techniques involving whole tissue homogenation and fractionation by centrifugation. In this study x-ray microanalysis of quick-frozen liver tissue, cryo-sectioned for the electron microscope, provided an alternative approach to studying subcellular ion distribution.

Based on x-ray microanalysis of mitochondria and nuclei, three populations of parenchymal cells were identified in the livers of mice treated with carbon tetrachloride. One group could not be readily distinquished from cells of control animals, another group of cells had mitochondria with high density calcium granules (as has been reported by others), and a third group had mitochondria which were similar to control in their elemental compositions but had substantially higher (4x) concentrations of chlorine in the nuclei. In some cases all three types of cells were present in the same thin section. Neither high calcium mitochondria nor high chlorine nuclei could be found in any of the cells of control animal liver tissue including cells in sections showing obvious freeze-damage.

Male CD-1 ICR mice obtained from Charles River Breeding Laboratories, Wilming ton, MA, were given 80 mg/kg of carbon tetrachloride (0.5% of the reported LD_{50}) in paraffin oil by oral gavage and sacrificed by decapitation 20 hours later. Immediately after sacrifice four 1 mm cubes shaped like pyramids were taken from the interior of the central liver lobe of each animal. Each cube was mounted on a silver pin with a small drop of glycerol and plunged into a slush of 10% isopentane in liquid propane. The temperature of the mixture was -182 °C. Pieces of whole liver lobe were also successfully prepared by the freezing plier technique.[2] However, although cells on the periphery of the liver lobe showed good cryo-fixation, cells in the interior of the lobe showed evidence of freeze-damage and could not be used in our study.

The cryo-preserved liver samples were sectioned in a Reichert FC4 ultramicrotome with cryo-attachment. Temperature settings were knife = -121°C and specimen = -122°C. The measured temperature at the knife/specimen interface was -133°C. Thin sections (3000 A) were placed on carbon-stabilized formvar-coated grids, pressed and transferred to the JEOL 100CX electron microscope in the frozen state. They were then freeze-dried in the microscope vacuum. The sections were viewed in the JEOL 100CX at an accelerating voltage of 100 KV. Examples of freeze-dried unstained sections of liver cells are shown in Figures 1 and 2.

X-ray spectra (200 seconds) were collected from 0.1 μm^2 areas within mitochondria, nuclei, or other subcellular components using an ORTEC Si-Li detector and EEDS II multichannel analyzer. Spectra were transferred via floppy disk to a PDP 11/70 computer. Peak-to-continuum values for each

element were determined using a version of the Sequential Simplex program developed at the National Institutes of Health.[3] The Fortran IV Plus program was run in the IAS Operating System and required 56K bytes of memory. A plot of the best-fit gaussian curve overlayed on the original data was produced for each spectra using Calcomp-Tektronix plotting routines. Normalized Chi-Squared values for each elemental peak were routinely close to one. A standard curve constructed with binary crystalline salts was used to convert peak-tocontinuum ratios to millimole per kilogram dry weight values.[4] The standard curve was calibrated with data from pure organic materials containing chemically bound ions of interest: glutathione (sulfur) and cyclohelamonium phosphate (phosphorus) (Sigma Chemical, St. Louis, MO). Corrections were made for mass loss with sulfur and chlorine.

Some tissue cubes were prepared by freeze-subsitution techniques in order to check the quality of the cryofixation. For freeze-substitution, frozen samples were transferred to pre-cooled (-80°C) tetrahydrofuran, (THF, Lot 744613, Fisher Scientific) containing 1% OsO_4 and 0.025% uranyl acetate[5] for a minimum of 70 hours. Following osmication the issues were brought to room temperature by 1 hour steps in -20°C and +4°C freezers, whereupon they were rinsed with acetone and embedded in Epon. Thick (2 µm) and thin sections were cut on LKB or Reichert ultramicrotomes. Phase contrast microscopy shown in Figures 3 and 4 was performed on a Wild research photoscope. The photomicrographs show both morphological differences for treated and control tissue and differences in the freezing preservation of the tissues.

Before liver cells can be differentiated on the basis of more subtle subcellular ion concentration differences information must be available about the normal concentration variability present within an individual liver cell. Figure 5 is a photomicrograph of one liver cell and its surroundings. In addition to the nucleus and mitochondria, some rough endoplasmic reticulum and a bile canaliculus are evident. Figure 6 shows a computer assisted drawing of the section and the results of the analyses for one element at 56 sites. This figure gives an indication of the variability in subcellular elemental concentrations within one cell. Data on the other elements detected at these sites and other similar cell analyses were used to develop parameters for statistically representative sampling.

REFERENCES

1. Schanne, F. A. X. et al., Science 206(1979)700.
2. Parsons, D. et al., EMSA Bulletin 14(1985)49.
3. Fiori, C. E. et al., NBS Spec. Publ. 604(1981) 233.
4. Shuman, H. et al., Ultramicroscopy 1(1976)317.
5. Phillips, T. E. and A. F. Boyner, J. Electron Micros. Tech. 1(1984)9.
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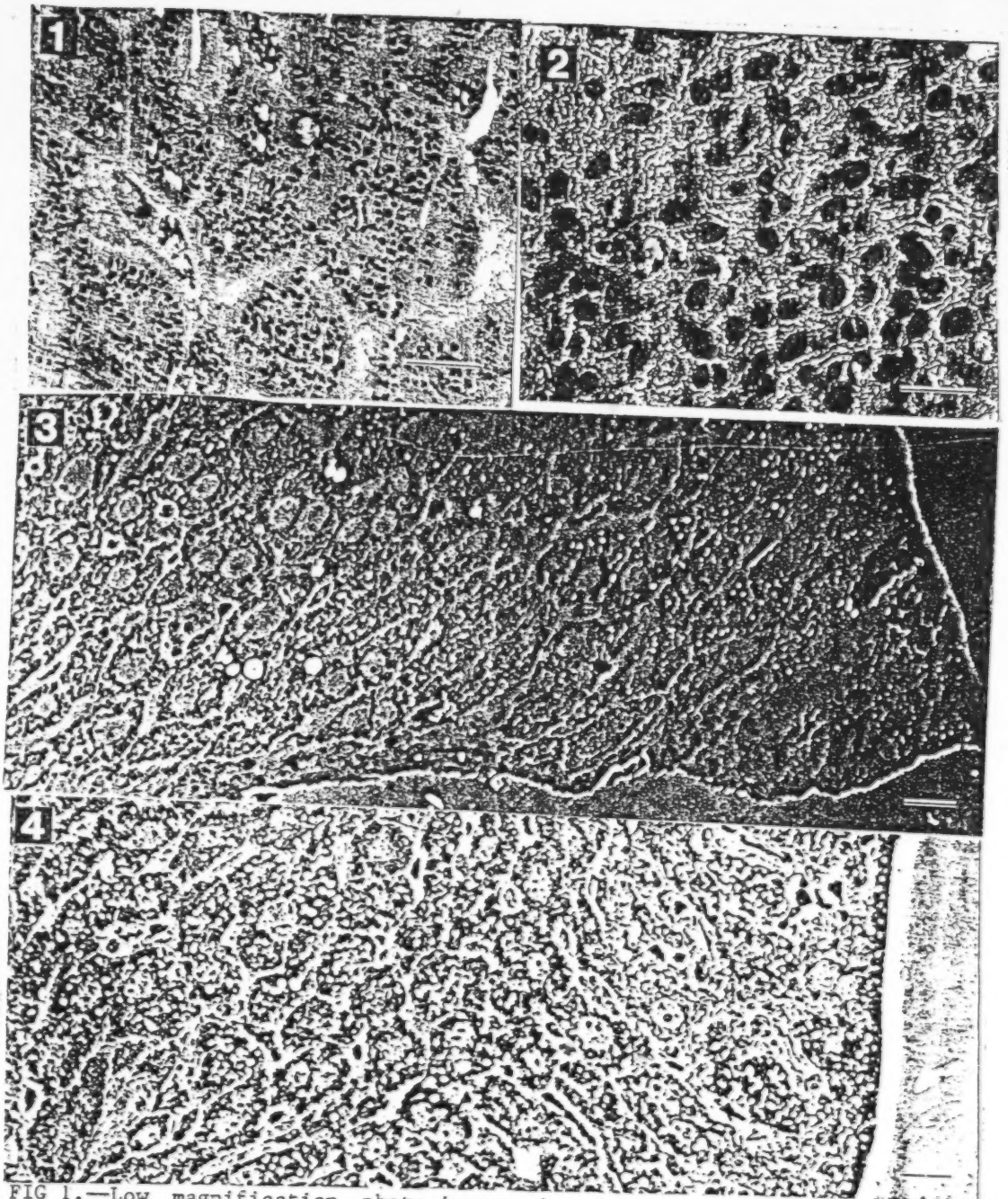

FIG 1.—Low magnification photomicrograph of an unstained, freeze-dried liver section. Nuclei, sinusoids, and mitochondria are visible. Bar equals 5 µm.

FIG 2.—Higher magnification showing mitochondria and rough endoplasmic reticulum. Bar equals 1 µm.

FIG 3.— Phase contrast photomicrograph of control mouse liver tissue which was frozen by plunging into isopentane/propane slush (-182°C). The surface cells exhibit atypical vacuolation and lack of good morphological preservation while deeper cells display good mitochondrial and cytoplasmic preservation. Bar equals 20 µm.

FIG 4.—Phase contrast photomicrograph of CCl_4-treated mouse liver tissue, frozen and prepared by methods identical to control tissue (Figure 3). Vacuolation typical of that associated with the effects of CCl_4 is present in deeper cells but the morphological preservation is the same as that shown in the control tissue. Bar equals 20 µm.

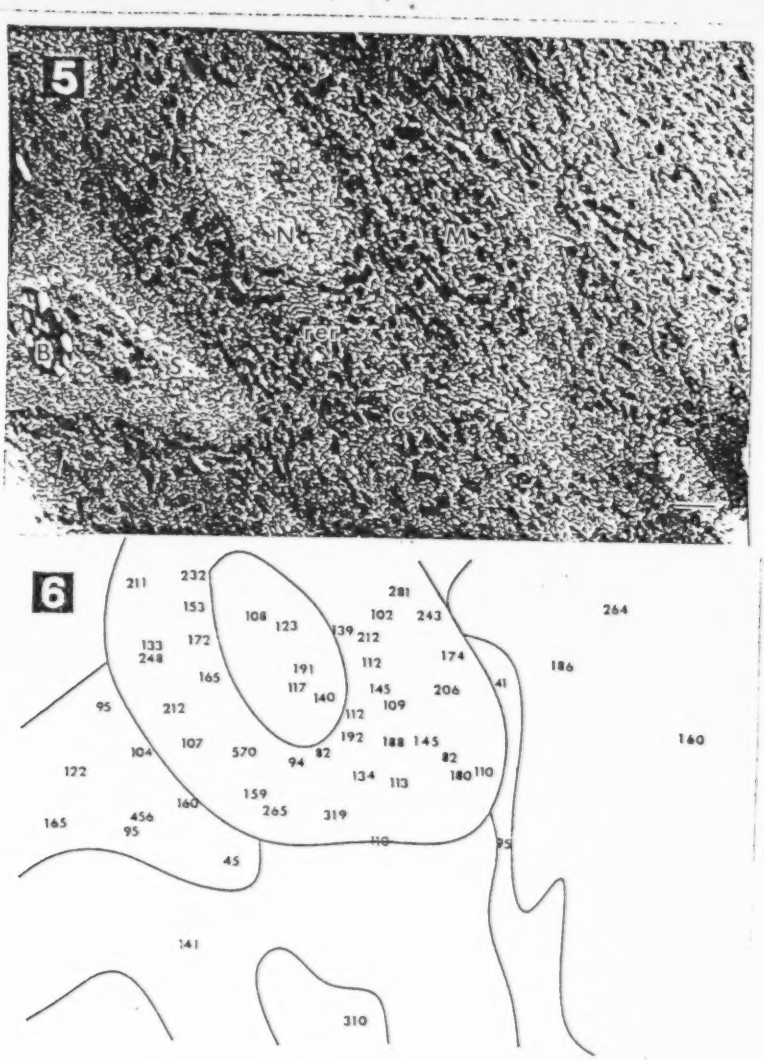

FIG 5.--Photomicrograph of a single parenchymal cell and surrounding area in unstained,, freeze dried liver tissue. The nucleus (N), mitochondria (M), rough endoplasmic reticulum (rer), bile canaliculus (C), sinusoid (S), and blood cell (B) are readily identifible. Bar equals 1 µm.
FIG 6.--Computer-assisted drawing of the cell in Figure 5 showing the location and magnitude of the sulfur concentration as determined by x-ray microanalyis at that spot. Values in millimole per kg/dry weight.